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PATENT

Case Docket No. GENAPP.002RA

Date: September 22, 1999

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ASSISTANT COMMISSIONER FOR PATENTS  
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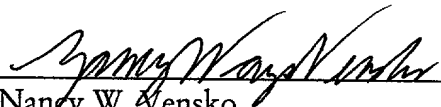
Sir:

Transmitted herewith for filing is the reissue application of

Inventor: T. Venkat Gopal  
For: PEPTIDE-MEDIATED GENE TRANSFER

Enclosed are:

- (X) A single-column copy of the specification, claims, and abstract
- (X) Copy of U.S. Patent 5,670,347
- (X) Reissue Application Declaration
- (X) Reissue Application By The Inventor, Offer To Surrender
- (X) Assent Of Assignee To Reissue, Establishment Of Right Of Assignee To Take Action And Revocation And Power Of Attorney
- (X) Title Report
- (X) Verified Statement (Declaration) Claiming Small Entity Status
- (X) Notice Regarding Litigation Involving Original Patent
- (X) Reissue Application Fee Determination Record
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Nancy W. Vensko  
Registration No. 36,298  
Attorney of Record

JC572 U.S. PTO  
09/22/99

Date: September 22, 1999

## REISSUE APPLICATION FEE DETERMINATION RECORD

## CLAIMS AS FILED - PART I

	CLAIMS IN PATENT	NUMBER FILED IN REISSUE APPLICATION	NUMBER EXTRA	RATE	FEE
BASIC FEE					\$380
TOTAL CLAIMS	14	26	12	\$9	\$108
INDEP. CLAIMS	3	16	13	\$39	\$507
TOTAL FILING FEE					\$995

## CLAIMS AS AMENDED - PART II

FOR	CLAIMS REMAINING AFTER AMENDMENT	HIGHEST NO. PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE	ADDITIONAL FEE
TOTAL CLAIMS		minus	=	\$9	\$
INDEP. CLAIMS		minus	=	\$39	\$
TOTAL ADDITIONAL FEE FOR THIS AMENDMENT					\$0

- (X) The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 11-1410. A duplicate copy of this sheet is enclosed.
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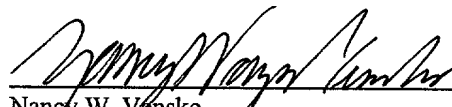
  
 Nancy W. Vensko  
 Registration No. 36,298  
 Attorney of Record

Figure 1 consists of 12 histograms arranged in a single column. Each histogram represents the frequency distribution of the number of non-zero elements in the vector  $x$  for a specific value of  $n$ . The x-axis for all histograms is 'Number of non-zero elements in  $x$ ' with major ticks at 0, 20, 40, 60, 80, 100, and 120. The y-axis is 'Frequency' with major ticks at 0, 2, 4, 6, 8, and 10. The histograms are labeled with  $n$  values: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120. As  $n$  increases, the distribution of non-zero elements becomes more concentrated around a central value, which appears to be approximately 60-70.

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL-ENTITY STATUS**

I, the undersigned, do hereby declare that:

[X] I am an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: GENETIC APPLICATIONS, LLC  
ADDRESS OF CONCERN: 11009 Alonda Court, San Diego, CA 92126

I further declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both. I further declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention described in the patent or application identified above.

The individual, concern or organization identified above has not assigned, granted, conveyed or licensed, and is under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

If the rights held by the above-identified individual, concern or organization are not exclusive, each individual, concern or organization having rights in the invention are identified below. Each such individual, concern or organization must file separate verified statements averring to their status as small entities.

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small-entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: David R. Staggs  
TITLE OF PERSON (if not an owner or individual): Chief Executive Officer

SIGNATURE: David R. Staggs DATE: 9/24/99

## PEPTIDE-MEDIATED GENE TRANSFER

## BACKGROUND OF THE INVENTION

The present invention is directed to a methodology for highly efficient, stable integration of DNA into a eukaryotic genome. More specifically, the present invention is directed to the use of a synthetic polypeptide, containing a nuclear localization signal, to complex with a DNA molecule and to facilitate its transportation and integration into the nuclear genome of a mammalian or other eukaryotic cell, for example, in the context of producing cell lines with an extended life.

DNA-CaPO<sub>4</sub> co-precipitation was the first method developed to introduce genes into mammalian cells. ("Gene" in this regard denotes a structural DNA segment, i.e., a DNA that codes for a polypeptide, and comprehends oncogenes as well as DNAs coding for a known expression product.) The co-precipitation method was applicable only to certain cell types, however, and could not be used to introduce genes into a wide variety of cell lines, especially those of hematopoietic origin. Moreover, the stable gene transfer efficiency was rather low, on the order of 10<sup>-4</sup> to 10<sup>-6</sup>. McNally, M. A., et. al., *BioTechniques* 6: 8826 (1988); Yen, T. S. B., et. al., loc. cit. 6: 413 (1988).

Limits on introducing and expressing genes in cultured mammalian cells motivated a search for other, more efficient approaches to gene transfer. Methods were developed, for example, that utilized chemical agents which were positively charged and, hence, able to complex with negatively charged DNA molecules. Examples of such agents include DEAE dextran and various cationic lipid molecules. Cells treated with DNA complexes comprised of such an agent can lead to the introduction of the DNA into different mammalian cell lines. Mannino, R. J. et. al., *BioTechniques* 6: 682 (1988); Felgner, P. et al., *Proc. Nat'l Acad. Sci. USA* 84: 7413 (1987); Fraley, R. et al., *Trend Biochem. Sci.* 6: 77 (1981); Holter, W. et. al., *Exp. Cell Res.* 184: 546 (1989); McCutchan, J. H. et al., *J. Nat'l Cancer Inst.* 41: 351 (1986); Chaney, W. C. et al., *Somatic Cell & Mol. Genet.* 12: 237 (1986).

The production of a gene product for only a short time period after transfection, usually from 48 to 72 hours, is called "transient expression." Many of the DNA-complexing agents reported heretofore, while useful in transferring a gene into mammalian cells, resulted in only transient expression of the introduced gene in a small fraction of the transfected cells. See, for example, Miller et. al., *Proc. Nat'l Acad. Sci., USA*, 76: 949 (1979); Oi et al., loc. cit. 80: 825 (1983).

In addition to giving poor results with respect to stable gene expression, transfer methods based on such DNA-complexing agents often were effective only with established cell lines, and did not work very well with primary cells isolated from various mammalian species. Other techniques therefore were needed to enhance gene transfer efficiency, to increase the variety of cell types capable of being transfected, and to effect stable gene transfer. Stable gene transfer is the ability of cells to maintain and express transfected DNAs in a stable manner, through integration of the transfected DNA into cell chromosomes.

Retroviral vectors, which were under development at about the same time seemed to be quite effective in transferring genes into different cell types. The use of such vectors was prompted by the elucidation of gene regulation in various murine and avian retroviruses. Two other developments led to the development of retrovirus-based gene transfer vehicles. The first development was the identification of minimal sequences required for efficient packaging of viral particles in a cell line which produced the coat proteins and other structural components of the viral particle in trans. The cell lines that provided the structural components for virus development are called "packaging" cell lines. The second significant step in the establishment of retroviral vectors was the development of both ecotropic and amphotropic packaging cell lines, which aided the design of recombinant retroviral particles which could infect both murine and human cell lines.

Additional modifications of retroviruses were deemed necessary to address concerns that retroviral vectors could recombine in vivo to generate wild-type virus. Developments in this regard yielded a number of safe retroviral vectors which have been used to transfer genes into a variety of established mammalian cell lines, as well as into certain primary cells in a few instances. E. Gilboa et al., *BioTechniques* 4: 504 (1986); A. D. Miller et al., *Mol. Cell. Biol.* 6: 2895 (1986); H. Stuhlmann et al., loc. cit. 9: 100 (1989); A. D. Miller et al., *BioTechniques* 7: 980 (1989); J. A. Zwiebel et al., *Science* 243: 220 (1989).

Even though these vectors were effective with respect to various mammalian cells, there were many restrictions on a wider application of the retroviral gene-transfer technique. These limitations included (1) the size of exogenous DNA that can be inserted into a retroviral vector and (2) the use of only dividing cells for retroviral gene transfer. E. Gilboa, *BioTechniques*, supra (1986); A. D. Miller, supra (1986); H. Stuhlmann, et al., *Mol. Cell. Biol.* supra (1986); A. D. Miller et al., *BioTechniques*, supra (1986); J. A. Zwiebel et al., supra (1989).

Other viruses have been used to generate recombinant viral vectors for gene transfer studies. Adenovirus, adeno-associated virus, herpes simplex virus, and even HIV have been employed as vectors to introduce genes into both established cell lines and primary cells. Some of these viral vectors are capable of transferring genes into non-dividing cells. R. J. Samulski, et al., *EMBO J.* 10: 3941 (1991); J. D. Tratschin, et al., *Mol. Cell. Biol.* 5: 3251 (1985); P. L. Hermonat, et al., *Proc. Nat'l Acad. Sci. (USA)* 81: 6466 (1984); D. J. Fink, et al., *Human Gene Therapy* 3: 11 (1992).

Viral vectors capable of transferring genes into non-dividing cells usually require the generation of high-titer viral stock in order to achieve high efficiency gene transfer into different cell types. In addition, whenever a different regulatory sequence is to be tested for optimal level of gene expression into primary cells, a new viral stock must be made and titered for every modification. All these involve very time-consuming experimental manipulations.

Still another concern relates to the application of viral vectors in human gene therapy. A number of studies have been carried out in primates to test the safety of retroviral vectors for introducing cells transduced with retroviral vectors into animals. Some of these animals have developed various forms of lymphoma. R. E. Donahue, et al., *J. Exp. Med.* 176: 1125 (1992). Additional safety features have been introduced into some of the newer versions of retroviral vectors, yet are not available for all types of viral vectors.

## SUMMARY OF THE INVENTION

It therefore is an object of the present invention to provide a method for high efficiency gene transfer to achieve expression, stable as well as transient, in a wide spectrum of cell types, including primary cells from various mammalian species.

It is also an object of the present invention to provide cell lines which, even if derived from primary mammalian cells, are characterized by an extended life in culture.

It is another object of the present invention to provide a readily implemented screening system for identifying sequences that influence in the expression of cloned genes in various primary cell types from different species.

In accomplishing these and other objectives, there has been provided, in accordance with one aspect of the present invention, a transfection vector comprising a synthetic polypeptide linked electrostatically to a DNA structural sequence, forming a polypeptide-DNA complex, where the polypeptide is comprised of (A) a polymeric chain of basic amino acid residues, (B) an NLS peptide and (C) a hinge region of neutral amino acids that connects the polymeric chain and the NLS peptide. The polymeric chain preferably is comprised of between 10 and 50 residues, which can be selected from lysine, arginine and ornithine, for example, while the hinge region is comprised of between 6 and 50 amino acid residues selected, for example, from glycine, alanine, leucine and isoleucine. The NLS peptide preferably is located at the amino terminus of said polypeptide and the polymeric basic amino acid chain at the carboxyl terminus. Among exemplary NLS peptides are the SV40 large T antigen NLS sequence, the polyoma large T antigen NLS sequence, the adenovirus E1a NLS sequence, and the adenovirus E1b NLS sequence.

In accordance with another aspect of the present invention an extended life cell line is provided that is the product of transfecting a mammalian cell with a vector as described above. The mammalian cell thus transfected can be selected, for example, from the group consisting of a human umbilical vein endothelial cell, a human dermal microvascular endothelial cell, a human peripheral blood monocyte/macrophage cell, a human aortic smooth muscle cell, and a rabbit liver non-parenchymal cell.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

# DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention allows for the highly efficient transfer and stable integration of DNA into eukaryotic cells, such as cells from established mammalian cell lines, primary cells from mammalian tissues, and plant cells. The present invention also can be applied to developing cell lines from non-dividing cells, such as human peripheral blood monocytes and macrophages.

In accordance with the present invention, a synthetic polypeptide is provided that can complex with a DNA molecule very efficiently by taking advantage of the high negative charge density on the polynucleotide. To this end, a synthetic polypeptide of the present invention comprises a DNA-binding sequence that is rich in basic amino acids, such as lysine, arginine and ornithine, and that is typically ten to fifty residues long. D-isomers of these basic amino acids are suitable so long as the length of the stretch of basic amino acids is within the prescribed length. The DNA-binding sequence can be a homopolymer of a basic amino acid, or it can comprise more than one kind of basic residue. The DNA binding sequence must be of adequate length to bind DNA, yet not so long that it precipitates out of the solutions employed in the present methodology, as discussed below.

























A synthetic polypeptide of the present invention also contains an amino acid sequence corresponding to a nuclear localization signal (NLS) sequence. A representative sample from the diverse range of nuclear localization signals which have been identified are listed in Table I below. (SEQ ID NOS:1-54).

TABLE I

Source	Nuclear Protein	Deduced Signal Sequence
Yeast	MAT $\alpha$ 2	(SEQ ID NO: 1) K-I-P-I-K (SEQ ID NO: 2) V-R-I-L-E-S-W-F-A-K-N-I
SV40	Large T	(SEQ ID NO: 3) P-K-K-K-R-K-V
Influenza virus	Nucleoprotein	(SEQ ID NO: 4) A-A-F-E-D-L-R-V-R-S
Yeast	Ribosomal protein L3	(SEQ ID NO: 5) P-R-K-R
Polyoma virus	Large T	(SEQ ID NO: 6) V-S-R-K-R-P-R-P-A
SV40	VP1	(SEQ ID NO: 7) A-P-T-K-R-K
Adenovirus	E1a	(SEQ ID NO: 8) K-R-P-R-P
SV40	VP2 (VP3)	(SEQ ID NO: 9) P-N-K-K-K-R-K
Frog	Nucleoplasmin	(SEQ ID NO: 10) R-P-A-A-T-K-K-A-G-Q-A-K-K-K-K-L-D
Rat	Glucocorticoid receptor	(SEQ ID NO: 11) K-K-K-I-K
Monkey	v-sis (PDGF B)	(SEQ ID NO: 12) R-V-T-I-R-T-V-R-V-R-R-P-P-K-G-K-H-R-K
Yeast	Histone 2B	(SEQ ID NO: 13) G-K-K-R-S-K-A
Chicken	v-rel	(SEQ ID NO: 14) K-A-K-R-S-K-A
Influenza	NS1	(SEQ ID NO: 15) D-R-L-R-R (SEQ ID NO: 16) P-K-Q-K-R-K
Frog	N1	(SEQ ID NO: 17) V-R-K-K-R-K-T (SEQ ID NO: 18) A-K-K-S-K-Q-E
Human	c-myc	(SEQ ID NO: 19) P-A-A-K-R-V-K-L-D (SEQ ID NO: 20) R-Q-R-R-N-E-L-K4S-F
Human	lamin A	(SEQ ID NO: 21) T-K-K-R-K-L-E
HIV-1	Rex(p27 <sup>src</sup> )	(SEQ ID NO: 22) P-K-T-R-R-R-P (SEQ ID NO: 23) S-Q-R-K-R-P-P
Adenovirus	pTP	(SEQ ID NO: 24) R-L-P-V-R-R-R-R-V-P
HIV-1	Tat	(SEQ ID NO: 25) G-R-K-K-R
Frog	Lamin L <sub>1</sub>	(SEQ ID NO: 26) V-R-T-T-K-G-K-R-K-R-I-D-V



TABLE 1-continued

Rabbit	Progesterone receptor	(SEQ ID NO: 27) R-K-F-K-K
HIV-1	Rev	(SEQ ID NO: 28) R-R-N-R-R-R-R-W
Human	PDGF A-chain	(SEQ ID NO: 29) P-R-3-S-G-K-K-R-K-R-L-K-P-T
Mouse	c-abl	(SEQ ID NO: 30) K-K-K-K-K
Adenovirus	DBP	(SEQ ID NO: 31) P-P-K-K-R
		(SEQ ID NO: 32) P-K-K-K-K-K
Chicken	c-erb-A	(SEQ ID NO: 33) S-K-R-V-A-K-R-K-L
Human	c-myc	(SEQ ID NO: 34) P-L-L-K-K-I-I-Q
Human	N-myc	(SEQ ID NO: 35) P-P-Q-K-K-I-K-S
Human	p53	(SEQ ID NO: 36) P-Q-P-K-K-K-P
Human	Hsp 70	(SEQ ID NO: 37) F-K-R-K-H-K-K-D-I-S-Q-N-K-R-A-V-R-R
Hepatitis B virus	Core protein	(SEQ ID NO: 38) S-K-C-L-G-W-L-W-G
Chicken	Ets1	(SEQ ID NO: 39) G-K-R-K-N-K-P-K
Yeast	Ribosomal protein L29	(SEQ ID NO: 40) K-T-R-K-H-R-G
		(SEQ ID NO: 41) K-H-R-K-H-P-G
Protein		Nuclear Localization Signals
TGA-1A (tobacco)		(SEQ ID NO: 42)  -L-A-Q-N-R-E-A-A-  -S-R-L- 
TGA-1B (tobacco)		(SEQ ID NO: 43)  -A-R-L-V-R-N-R-E-S-A-Q-L-S
		(SEQ ID NO: 44)  -Q- 
O2 NLS B (maize)		(SEQ ID NO: 45)  -E-S-N-R-E-S-A-  -S-  - 
Nla (Polyvirus)		(SEQ ID NO: 46)  -N-Q-K-H-K-L-K-M-32aa 
VirD2 (Agrobacterium)		(SEQ ID NO: 47)  -P-R-E-D-D-D-G-E-P-S-E-  -E- 
VirE2 NSE1 (Agrobacterium)		(SEQ ID NO: 48)  -R-P-E-D-R-Y-I-Q-T-E-  -Y-C- 
VirE2 NSE2 (Agrobacterium)		(SEQ ID NO: 49) K-T-K-Y-G-S-D-T-E-I-K-L-I-S-K
O2 NLS A (maize)		(SEQ ID NO: 50) M-E-E-A-V-T-M-A-P-A-A-V-S-S-A-V-V-G-D-P
		(SEQ ID NO: 51) M-3-Y-N-A-I-L-  -L-E-E-D-L-E
R NLS A (maize)		(SEQ ID NO: 52) G-D-  -A-A-P-A-  -P
R NLS M (maize)		(SEQ ID NO: 53) M-S-E-  -E-K-L
RNLS C (maize)		(SEQ ID NO: 54) M-I-S-E-A-L-  -A-I-G- 

See Garcia-Bustos et al., *Biochem. Biophys. Acta* 1071: 83 (1991), Raikhel, N., *Plant Physiol.* 100: 1627 (1992), and Citovsky, V. et al., *Science* 256: 1802 (1992), the contents of each of which are hereby incorporated by reference.

In the present invention, an NLS peptide, which typically is six to fifteen amino acids in length, facilitates transport of the associated DNA into the nucleus. Because the synthetic polypeptide promotes the transport of the transfected gene into the nucleus of the host cell, this method provides both highly efficient stable and transient gene expression. Once inside the nucleus, the introduced DNA is immediately available to the transcription machinery, and can be expressed transiently. Simultaneously, the introduced DNA is also in the process of getting integrated into the host chromosome to give rise to stable expression. Thus, the method of the instant invention can achieve both transient and stable expression of introduced DNA.

Transient gene expression results when the method of gene transfer results in the introduction of the DNA sequences into the nucleus in a non-integrated form. Transient transfection is measured 24 to 72 hours after transfection by assays that measure gene expression of the transfected gene(s). In contrast, stable expression of the encoded protein results when the transferred DNA sequences are stably integrated into the chromosomal DNA of the target cell. Stable transfectants remain capable of expressing the transfected DNA after two weeks or greater following the method of the invention. Commonly used assays monitor enzyme activities of chloramphenicol acetyltransferase (CAT), LAC-Z,  $\beta$ -galactosidase ( $\beta$ -gal),  $\beta$ -glucuronidase

(GUS), luciferase, or human growth hormone, each of which may be contained in the present invention.

The NLS domain of the synthetic peptide is based on known endogenous peptide sequences that were identified by reference to two criteria: (1) sufficient to redirect a cytoplasmic protein to the nucleus and (2) necessary for directing a nuclear protein to the nucleus. Methods for assessing an NLS peptide's ability to direct protein to the nucleus are known in the art. See Garcia-Bustos, et al., supra. Sandler et al., *J. Cell Biol.* 109: 2665 (1989), and Citovsky et al., supra, the respective contents of which are hereby incorporated by reference. For example, an NLS peptide or a natural protein containing an NLS is fused to an otherwise non-nuclear protein, by either synthetic or recombinant production. The hybrid protein is then assessed for its ability to target the non-nuclear protein to the nucleus.

The presence of the non-nuclear protein in the nucleus can be determined by a functional assay or immunofluorescence. An illustrative assay entails the histochemical determination of a product produced by the non-nuclear protein, such as a colorimetric marker produced by  $\beta$ -gal or GUS. (A "colorimetric marker" includes an enzyme that can catalyze a reaction with a substrate to elicit a colored product which can be detected or measured by a variety of means, such as standard fluorescence microscopy, flow cytometry, spectrophotometry or colorimetry. "Immunofluorescence" relates to detecting the presence of the non-nuclear protein in the nucleus by means of an antibody specific for the targeted protein.)

In the past NLS peptides have been studied to assess their ability to target reporter proteins to the nucleus. Also, endogenous proteins containing an NLS, such as the VirD2 and VirE2 of *Agrobacterium*, have been shown to mediate the transfer of the *Agrobacterium* single-stranded DNA intermediate T-strand to the plant cell nucleus endogenously. See Citovsky, et al., supra. There has been no suggestion heretofore, however, to use an NLS peptide to target a polynucleotide to the nucleus of a eukaryotic cell.

A preferred NLS domain contains a short stretch of basic amino acids like the NLS of the SV40 virus large T antigen (PKKKRKV) (SEQ ID NO:3), which is an NLS that has been shown to be effective in mammalian cells (basic residues are highlighted). Another preferred NLS domain consists essentially of short hydrophobic regions that contain one or more basic amino acids (KIPK) (SEQ ID NO:1), which is like the NLS of mating type  $\alpha 2$ . The NLSs that transport DNA into the plant cell nucleus often are bipartite, which means that they are usually comprised of a combination of two regions of basic amino acids separated by a spacer of more than four residues (see stippled segments in Table I), such as the *Xenopus* nucleoplasmin (KRPAATKKAGQAKKKK) (SEQ ID NO:55).

The NLS peptide of the present invention can be designed to accommodate different host cells, both mammalian and plant cell hosts.

The method described here can suitably be modified to introduce genes into plant protoplasts using plant NLSs, such as those described by Raikhel (1989), supra.

The present gene transfer system is also capable of transferring foreign DNA into gymnosperms and angiosperms. Procedures for assessing the introduction of foreign DNA in plants are known to the art, such as those disclosed by Miki, B. L., et al., in *METHODS IN PLANT MOLECULAR BIOLOGY AND BIOTECHNOLOGY*, B. R. Glick et al., eds. (CRC Press, 1993), and Gruber, M. Y. et al., id.

A synthetic polypeptide of the present invention thus is comprised of a DNA binding domain and an NLS peptide domain which are separated by a third element, a hinge region of neutral amino acid, to minimize steric interference between the two domains. For this purpose, the hinge region ranges in length from about six to twenty-five amino acids, and contains a stretch of neutral small amino acids without any bulky hydrophobic or ionic side chains.

The NLS sequence can be located at either the amino terminus or the carboxy-terminus of the synthetic peptide. The arrangement of the two domains, basic amino acid sequence and NLS sequence can be interchanged without affecting the high gene transfer efficiency. As indicated previously, such a synthetic polypeptide binds electrostatically to the DNA that is to be introduced into the target cell. The weight ratio of polypeptide to DNA in the resulting complex generally is in the range of 1:1 to 1:10; for example, 1 µg polypeptide to 1 to 10 µg of polynucleotide.

In accordance with the present invention, entry of the DNA-polypeptide complex into cells can be promoted by treating target cells with a hypertonic solution, followed by hypotonic treatment of cells in the presence of gene-peptide complex. See, for example, Okada and Rechsteiner, *Cell* 29: 33 (1982). A suitable hypertonic solution can contain both polyethylene glycol (PEG) and sucrose, preferably in the concentration of 0.3M-0.6M and 10% to 25%, respectively, and is referred to as "primer" hereinafter. Okada et al., *supra*, and T. Takai, et al., *Biochem. Biophys. Acta* 1048: 105 (1990).

The methodology of the present invention has been used to develop stable transfectants of different established cell lines. It also has been employed to transfer genes into primary cells from different mammalian species, thereby to obtain cell lines that retain many of the characteristics of the cognate primary cells. Cell lines developed from primary cells via the methodology of the present invention are called "extended life" cell lines in this description, because the cell lines so developed retain almost all of the characteristics of their cognate primary cells even in their late passage. The range of cell types that can be converted to extended life cell lines, according to the present invention, is based on the availability of primary cells or the ability to isolate a primary cell from the organ in question. In this regard, the inventive methodology is not limited to cell types amenable to transformation. In addition to the cell types already mentioned, the present invention can be applied to pancreatic beta cells, human liver and kidney cells, and human hematopoietic stem cells, among others.

The methodology of the present invention has been used to develop an extended life cell line from human monocyte/macrophage cells, which are normally non-dividing. In all these instances, stable cell lines were obtained with a very high efficiency, either comparable to or better than the efficiency using retroviral vectors.

The present invention finds application as well in both ex vivo and in vivo gene therapies, where genetic material is transferred into specific cells of a patient. Ex vivo gene therapy entails the removal of the relevant target cells from the body, transduction of the cells in vitro, and subsequent reintroduction of the modified cells into the patient.

A gene therapy pursuant to the present invention could involve an ex vivo introduction, into a particular cell type from the patient, of a polynucleotide coding for a correcting protein which can be produced in functional form by the targeted cell type. Genes suitable for expression in this regard include an adenosine deaminase gene, a globin gene, an LDL receptor gene, and a glucose cerebrosidease gene.

Different kinds of gene-therapy applications require either stable or transient gene expression. The method of the present invention is advantageous in that it can be used in gene therapy requiring either stable gene expression or transient gene expression. Transient expression of a foreign gene is preferred when expression of the exogenous product is needed only for a short period of time; thereafter, rapid clearance of the gene product and its vector is desirable. Transient expression is also desirable when the prolonged effects of the exogenous protein's expression are unknown. Stable expression in gene therapy is needed when the patient has a genetic defect that is incompatible with life. Such genetic defects include but are not limited to cystic fibrosis, Tay Sachs and cancer. Mulligan, *Science* 260: 926 (1993).

A gene therapy pursuant to the present invention also could involve an in vivo introduction of a structural DNA into cells of a patient's body. For stable transfer of genes into a target tissue using this method, the ligand to the target receptor will be conjugated to the synthetic polypeptide. The polypeptide-ligand combination can be complexed to a polynucleotide coding for the needed protein and then introduced into the host organism through blood circulation. When this complex reaches the target tissue, the whole complex will be taken up by cells containing the corresponding receptor for the ligand through receptor mediated process. Because of the NLS in the polypeptide-ligand complex, the complex will enter into the nucleus, resulting in a stable integration of the introduced gene into the host chromosome and, thereby, a correction of the genetic defect in the host. Cell-specific receptors are well known to those of skill in the art, as are their ligands which can be used in complexes for receptor-mediated gene transfer. Michael, S. L. et al., *J. Biol. Chem.* 268: 6866 (1993). For example, when the liver is the tissue targeted for gene therapy, the DNA encoding corrective protein is complexed to a synthetic neoglycoprotein that will target the complex to the asialoglycoprotein receptor on hepatocytes. For example, a cell type specific receptor such as asialoglycoprotein can be chemically linked to the transfection vector at the carboxyl terminal of the synthetic polypeptide molecule to deliver the foreign gene directly into liver cells. An additional hinge region can be incorporated into the molecule before chemically linking the polypeptide molecule to a cell-type specific ligand molecule, such as asialoglycoprotein or a cell-specific monoclonal antibody.

An example of a carrier useful for receptor-mediated gene transfer to liver is a synthetic glycoprotein in which bovine serum albumin (BSA) is covalently bound to poly L-lysine using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). Ferkol et al., *FASEB* 7: 1081 (1993). To produce a neoglycoprotein conjugate for use in targeting DNA to liver, a reaction mixture that contains about 170 mM galactose, 4 mM poly (L-lysine), 160 mM BSA and 10 mM EDC (pH 7.5) can be incubated for 48 hours at 22° C. DNA is complexed to the neoglycoprotein carrier in a 360:1 molar ratio. The carrier-DNA complexes are dialyzed against 150 mM sodium chloride before transfection.

Expression of a functional protein after transfection with DNA complexed to ligand alone is often transient. Ferkol et al., *supra*. The method of the present invention greatly improves the cell-specific targeting of receptor-mediated transfection by providing stable expression by increasing stable integration of a foreign DNA in the host cell using a synthetic polypeptide molecule of the present invention.

A variation of receptor-mediated gene transfer employs coupling a synthetic polypeptide as described above to monoclonal antibodies which recognize a cell surface antigen on the target cells. Maruyama et al., *Proc. Nat'l Acad. Sci. USA* 87: 5744 (1990). The coupled monoclonal antibody and synthetic polypeptide then are complexed with a DNA encoding the required or desired protein. This complex will target the DNA to the cells expressing the corresponding cell surface antigen. Any tissue of the human body can be targeted for the gene therapy of the present invention using the disclosed methods. A target tissue is suitable in this context so long as it is susceptible to genetic modification according to the present invention.

The present invention is further described with reference to the following examples, which are only illustrative and not limiting of the invention.

#### EXAMPLE 1

##### Transfer of Genes into Established Cell Lines for Purposes Transient Gene Expression and Selection of Stable Transfectants, Respectively

##### Forming the DNA-Polypeptide Complex and Transfecting Cells Therewith

The DNA or other polynucleotide to be transfected, such as a plasmid containing a gene for a drug resistance marker or coding a protein needed for expression in the host cell, is complexed to a synthetic polypeptide molecule in different weight ratios in an isotonic buffer solution. For example the weight ratio of DNA:polypeptide can be between 1:1 and 10:1, although ratios outside of this range may be evaluated empirically for achieving the objects of the present invention. An isotonic buffer solution such as Hanks buffered salt solution or HEPES buffered saline may be used for complexing DNA to polypeptide.

While the complex is formed, the cells that are to be transfected either remain attached to a substratum, such as a tissue culture dish, or are pelleted (for cells that grow in suspension). The cells are treated with a hypertonic primer solution, such as a concentration of 0.3M-0.6M sucrose and 10% PEG in either Tris-HCl or HEPES (pH 7.2) buffered solution, for 3-5 mins at room temperature. The primer solution then is removed.

After the DNA-polypeptide complex is formed, it is made hypotonic. The complex solution is hypotonic when it has a lesser osmotic pressure than a 0.15M or 0.9% solution of NaCl. For example, the complex in isotonic buffer can be made 40-55% hypotonic or 0.075M simply by adding an amount of distilled water that is equal to the volume of the complex in isotonic buffer. The hypotonic complex solution then is added to the cells that have been treated with the primer solution. Cells remain in the hypotonic DNA-polypeptide solution for 3-4 minutes. Fresh medium then is added to the cells to rinse away excess DNA-polypeptide solution. Thereafter, the cells are grown normally.

##### Producing a Synthetic Polypeptide Molecule

An example of an synthetic polypeptide molecule of the present invention is one consisting of the amino acid sequence PKKKRKVSGGGGKKKKKKKKKK(SQ ID NO:56). Such a peptide can be synthesized, using standard methods of peptide production, and purified by standard methods using high pressure liquid chromatography (HPLC).

### Selection of Stably Transfected Cell Lines

Transfected cells are grown in regular growth medium for 48 hours, and then plated in selective medium containing 400 µg/ml of G418. Cells were plated at a density of 100–1000 cells per 60 cm<sup>2</sup> dish. The number of G418-resistant colonies was determined two weeks after the initiation of selection. Other selectable markers, such as pHyg, may be used to achieve the results of the instant invention. K. Blochliger, et al., *Mol. Cell. Biol.* 4: 2929 (1984).

This method gave a stable-transfection efficiency of 5–10%. Similar results were obtained using either G418 or hygromycin selection. In general the stable transfection efficiency achieved by the method of the instant invention is a few orders of magnitude greater than prior art methods. The instant invention's 5–10% efficiency is several orders of magnitude better than the efficiency of the DNA-CaPO<sub>4</sub> co-precipitation method and at least equal or 5 times greater than the fairly high 1–10% level of stable transfection efficiency achieved by viral based methods.

TABLE II

Method	Stable Transfection Efficiency
Peptide-Mediated Gene Transfer	5–10%
Retroviral vectors	1–10%
Non-viral methods (e.g., CaPO <sub>4</sub> )	<2%

That the transfectants of the instant invention are stable is shown by the following example. When G418 resistant colonies were grown without selection for variable period of times, and then tested for resistance to the antibiotic by plating the cells under clonal conditions, the same number of colonies were obtained both with and without G418. This result indicates that, once the cells are selected for the expression of the Neo gene, the resistance gene was retained stably in the chromosome.

Three different cell lines were used to test the efficiency of gene transfer of the new method. Mouse fibroblast cell line (L cells), mouse erythroleukemia cell line (C19TK), and COS cells. The COS cell line was used to establish conditions for transient gene expression. The eukaryotic expression vector, CH110, contains bacterial β-gal and was employed in these studies. The β-gal gene in CH110 is under the control of SV40 virus early promoter.

The COS cells were treated with primer and then exposed to DNA-polypeptide (2.5–5.0 µg) complex under hypotonic conditions. After this treatment, cells were returned to the normal growth condition. Transfected cells were grown at 37° C. for 48 hrs, and stained for the expression of the β-gal reporter gene. Forty to fifty percent of the cells were positive for the expression of the reporter gene.

Mouse L cells were transfected with eukaryotic expression vector containing the Neo gene, which codes for the antibiotic G418 resistance gene. L cells are sensitive to G418 at 400 µg/ml. Cells plated in 24-well tissue culture plates were then transfected with synthetic polypeptide complexed to the plasmid pRSV-Neo via the methodology of the present invention.

A mouse erythroleukemia cell line, C19TK, also was used as a representative cell line for testing the transfection efficiency of the present invention with respect to hematopoietic cells. The expression vector, pDR2, which carries a hygromycin-resistance gene, was used for these studies. C19TK cells are exquisitely sensitive for the antibiotic hygromycin. This cell line grows in suspension and, hence, was transfected in suspension.

Briefly, about million cells are spun down and the cell pellet is treated with primer. The cells are then exposed to DNA-polypeptide complex under hypotonic condition. Forty-eight hours after transfection, a known number of cells are plated in microtiter plates with hygromycin. The number of wells with growing population of cells was enumerated to determine the transfection efficiency. The stable transfection efficiency was about 1-5%, as compared to most of the other non-virus-based methods that are very poor. Thus, the method described herein is very efficient for stable transfection efficiency both for hematopoietic and non hematopoietic cell lines. Only some retrovirus based vectors give a transfection efficiency comparable to the efficiency obtained with the current method for hematopoietic cell lines. See Gilboa, et al. (1986), Miller, et al. (1986), Stuhlmann, et al. (1989), Miller, et al. (1989), and Zwiebel, et al. (1989), each cited above.

## EXAMPLE 2

### Transfer of Genes into Human Primary Cells

The gene transfer method of the present invention was used to generate extended life cell lines from different human primary cells. Most of the primary cells have a limited in vitro life span. The following cell types were employed to test the efficacy of the inventive method to generate extended-life cell lines by transfer of various oncogenes, either singly, in pairs of combinations, or combinations of more than two oncogenes. Rhim, J. S., et al., *Oncogene* 4: 1403 (1989).

## EXAMPLE 3

### Production of Extended Life Cell Lines

The method of introducing genes into primary cells is the same as that described above for introducing genes into established cell lines, such as the mouse fibroblast cell line L cells and the mouse erythroleukemia cell line C19TK. The main difference is that the host cell is a primary cell isolated from different species, human or other mammalian species, and the primary cells have only a limited in vitro life span. The isolation of primary cells from various tissue sources are well known to those of skill in the art.

In order to extend the life of primary cells that are endogenously incapable of extended growth in vitro, the cells are transfected with different oncogenes, such as SV40 large T antigen, polyoma large T antigen, adenovirus E1A and E1B, v-fms, Bcl2, myc and ras. The oncogenes can be used either alone, in pairs of various combinations, or in combinations of more than two oncogenes.

In addition, other genes that do not come under the category of oncogenes may be used. For example, genes that are important for DNA synthesis and normally active during the S phase of the cell cycle, such as the dihydrofolate reductase gene (DHFR), thymidine kinase gene, thymidylate

synthetase gene, a DRTF1/E2F transcription factor encoding DNA, or DNA encoding the E2F transcription factor can be complexed to synthetic polypeptide and used to extend the life of primary cells. The human DHFR gene complexed to synthetic polypeptide can be introduced into primary cells to produce extended life cell lines. DNA encoding a transcription factor that is active during the S phase of the cell cycle are particularly useful in the method of the instant invention. La Thangue, N. B. *Trends in Biochemical Sciences* 19: 108 (1994); Johnson, D. G. et al., *Nature* 365: 349 (1993), the respective contents of which are hereby incorporated by reference.

Because untreated primary cells have only a limited life span in vitro, their ability to grow continuously in culture after treatment with the present invention served to select for extended life cell lines. No other drug selection markers need to be used to select for extended life cell lines derived from primary cells.

To produce extended life cells lines from primary cells, newly cultured primary cells were treated by the method of the present invention employing synthetic polypeptide conjugated with various oncogenes, such as SV40 large T antigen and/or Adeno E1A. The treated cells were plated in their appropriate growth media and passed after the cells reached confluency. A parallel set of a control untreated primary cells were cultured under the same growth conditions. Typically, control primary cells stop growing after about 4-10 passages, depending upon the cell type (cell split ratio was usually 1:4 by surface area). In contrast, continuously growing cell lines were obtained from different primary cell types described in the following examples.

#### EXAMPLE 4

##### Analysis of Transformed (Extended Life) Cells

Extended life cell lines containing the oncogene are identified by restriction cleavage, Southern analysis and/or Northern analysis using appropriate DNA probes.

The DNA of each transformed extended life cell line is analyzed by Southern hybridization to determine whether the cell lines carry the oncogenes used to establish such extended life cell lines. DNA is extracted from the cell lines and the nucleic acid pellet is re-suspended in 200  $\mu$ l of 10 mM Tris-Cl pH 7.4, 0.1 mM EDTA, and 10  $\mu$ g is digested with a specific restriction enzyme, electrophoresed through 1.0% agarose, and transferred to nitrocellulose. Southern, *J. Mol. Biol.* 98: 503 (1975). Filters are hybridized to a radioactively labelled DNA, encoding each of the oncogenes that gave rise to the corresponding extended life cell line, in the presence of 10% dextran sulfate. After overnight hybridization, the filters were washed twice in 2 X SSC, 0.1% SDS at 64° C.

Each transformed extended life cell line is analyzed by Northern hybridization to determine whether the cell lines transcribe the oncogenes. Cells not containing the oncogene of interest will not demonstrate transcripts in a Northern analysis whereas cells containing the DNA of interest will demonstrate a detectable transcript. Also, an ELISA method was used to detect the presence of oncogene products in some of the extended life cell lines, using publicly available antibodies that recognize the corresponding oncogene protein.



The presence of SV40 large T antigen and adenovirus E1A gene products in the HUVEC extended life cell line, as detected by ELISA, are shown in table 2. Briefly, the cell line grown in a 96 well tissue culture plate is fixed with glutaraldehyde and paraformaldehyde. The cells are then treated with antibodies to the corresponding oncogenes. Thereafter, the cells are washed and then treated with a secondary antibody linked with to  $\beta$ -galactosidase. The cells are washed and then treated with a substrate for  $\beta$ -galactosidase. The reaction develops a product which is then measured using a microplate reader.

To determine whether the extended life cell line has maintained the parental cell line phenotype may be determined by a number of ways. Extended life cells lines containing the oncogene are assessed by Northern analysis using a DNA probes encoding a cell-specific protein. The cell-specific DNA probe is labeled with  $^{32}\text{P}$ -dCTP by nick translation pursuant, for example, to Rigby et al., *J. Mol. Biol.* 113: 237 (1977). Northern hybridization indicates that the extended life cell line is capable of transcribing the cell-specific protein.

Also, the maintenance of the parental phenotype in cells lines established according to the present invention can be determined by a number of biochemical methods, such as ELISA and enzyme assays, that determine the presence or function of a protein specific to the parental cell line. An antibody recognizing a protein produced only by the parental cell line can be used in an ELISA or immunofluorescence assay. Cell-specific markers are well known to those of skill in the art. For example, albumin is a marker for hepatocytes, insulin is a marker for pancreatic beta islet cells, factor VIII is a marker for endothelial cells, actin and myosin are markers for smooth muscle cells, and non-specific esterase is a marker for brain microglial cells. In Table II, the parental phenotype of the extended life endothelial cells produced by the present method of the invention was verified by several ELISAs to determine the expression of cell-specific endothelial markers. The parental phenotype of the monocyte/macrophage extended life cell lines produced by the present method was verified using a lysozyme enzyme assay to measure macrophage specific markers.

## EXAMPLE 5

### Human Umbilical Vein Endothelial Cells

Endothelial cells isolated from the human umbilical vein can only be cultured for a limited of passages, usually five to six. These cells were transfected with a combination of oncogenes, SV40 large T antigen and adenovirus E1A, or with another combination of genes. At least two oncogenes are needed to develop a truly transformed cell line. Ruley, H. E., et al., *Nature* 304: 602 (1983). For the instant invention, SV40 large T antigen combined with v-myc or ras or some an other oncogene can be used. When the gens encoding SV40 large T antigen is combined with either adenovirus E1A or E1B genes in the method of the instant invention, extended life cell lines may be produced from human umbilical vein endothelial cells. E1A or E1B or SV40 large T antigen alone did not give rise to established cell line with the high frequency obtained from using SV40 large T antigen in combination with E1A or E1B. Synthetic polypeptide complexed to DNA encoding either the SV40 large T antigen or polyoma large T antigen combined with the E2F1 transcription factor gens also produces extended life HUVEC cells lines.

Since the non-transfected primary cells normally grow in vitro only for a limited population doublings, cells that have taken up the oncogenes capable of generating extended life span cell lines were selected simply by repeated passage of the cells. When the transfected population of cells grows continuously, as compared to a control population of parental cells, it is reasonable to conclude that the oncogenes used are capable of generating extended life cells from a given cell type.

In HUVEC, for example, SV40 large T antigen and adenovirus E1A or E1B were effective in giving rise to a cell line. This cell line has now been growing in culture for 40 passages. In contrast, normal HUVECs stop growing by passage 7 or 8. Such cell lines arose with a high efficiency. It also is possible to generate cell lines using as few as a couple of hundred cells, grown either in a 24- or 48-well plate. These cells also have the same morphological appearance as the primary HUVEC and also display many of the biochemical properties characteristic of normal HUVEC.

Some of the properties that are characteristic of endothelial cells that were measured in the HUVEC extended life cell line are also listed in Table 2. These properties were also measured by ELISA using specific antibodies listed in the Table 2.

TABLE III

ELISA assay for the expression of ELAM-1, VCAM-1, ICAM-1, SV40 large T antigen and adenovirus E1A by extended life HUVEC line		
Antibody	O D <sub>492</sub>	
	-IL-1	+IL-1
Control	0.071	0.069
Anti ELAM-1	0.212	1.016
Anti VCAM-1	0.146	0.520
Anti ICAM-1	0.422	1.524
Anti SV40 large T	0.618	—
Anti E1A	0.725	—

#### EXAMPLE 6

##### Human Cord Blood-Derived Monocyte Cell Line

Adherent cells from human cord blood cells were transfected with different combinations of oncogenes in suspension using the method of the present invention. The resulting cells are selected in Granulocyte-Macrophage Colony Stimulating Factor (G-CSF). Control cells did not grow in culture, whereas growing populations of monocytes were obtained with several combinations of oncogenes. One preferred combination of polyoma large T antigen and adenovirus E1B encoding DNA produced extended life monocyte cells lines with somewhat higher efficiency than other combinations. Another preferred combination of SV40 large T or polyoma large T antigen and the E2F1 transcription factor gene produces monocyte extended life cells with high efficiency. The monocyte extended life cells also display many of the properties of normal monocytes, which illustrates the utility of the present invention in generating cell lines of hematopoietic origin.

## EXAMPLE 7

### Extended Life Human Aortic Smooth Muscle Cells

The method of the instant invention has also been used to generate extended life cell lines using a specific combination of oncogenes. Human aortic smooth muscle cells were obtained from Clonetics Corporation (San Diego, Calif. U.S.A.) and transfected with several combination of oncogenes. The combination of polyoma large T antigen and E1B gave rise to a continuously growing population of smooth muscle cells. Another preferred combination of SV40 large T or polyoma large T antigen and the E2F1 transcription factor gene produces extended life human aortic smooth muscle cells with high efficiency. This cell line resembles the early passage primary aortic smooth muscle cells morphologically. The extended life human aortic smooth muscle cells also express smooth cell actin and myosin well beyond passage 20.

## EXAMPLE 8

### Other Extended Life Cell Types

Primary cells from other species, such as rabbit and monkey, also have been used to generate cell lines. Transfection methods employed for primary cells from non-human species are similar to those used for human primary cells. When developing an extended life cell line from a new primary cell, several different combinations of available oncogenes should be tried. For example, at least five or six pairs of combinations of SV40 large T antigen, adenovirus E1A, adenovirus E1B, polyoma virus large T antigen or others available to those in the art. That combination of genes that gives rise to an extended life cell lines from a given primary cell type is determined as described in the above examples.

When the E2F1 transcription factor gene is complexed to synthetic polypeptide in combination with DNA encoding either the SV40 large T antigen or polyoma large T antigen, extended life cell lines can be produced from a variety of primary cell types, such as HUVEC, dermal microvascular endothelial cells, human aortic smooth muscle cells, and bone marrow monocyte/macrophage cells. Thus, the method of the present invention can identify a combination of oncogene DNAs that is highly efficient in producing extended life cell lines from the primary cells of various species. The present invention also comprehends a combination of an oncogene and an S-phase transcription factor gene which likewise is highly efficient in producing extended life cell lines from different types of primary cells.

## EXAMPLE 9

### Identification of Cell Type-Specific Transcriptional and Translational Regulatory Sequences

The present invention provides a screening system for identifying sequences that influence the expression of cloned genes in various primary cell types from different species. The instant invention can identify cell type specific transcription and translational regulatory sequences. The sequence in question typically will be cloned into a vector containing a reporter gene, such as chloramphenicol acetyl transferase or luciferase, and then transfected into various cell types using the method described herein. Expression of the reporter gene determines the tissue specificity of the regulatory sequence.

# SEQUENCE LISTING

## ( 1 ) GENERAL INFORMATION:

( i i i ) NUMBER OF SEQUENCES: 56

## ( 2 ) INFORMATION FOR SEQ ID NO:1:

### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 5 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Lys Ile Pro Ile Lys  
1 5

## ( 2 ) INFORMATION FOR SEQ ID NO:2:

### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 12 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Val Arg Ile Leu Glu Ser Trp Phe Ala Lys Asn Ile  
1 5 10

## ( 2 ) INFORMATION FOR SEQ ID NO:3:

### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 7 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Pro Lys Lys Lys Arg Lys Val  
1 5

## ( 2 ) INFORMATION FOR SEQ ID NO:4:

### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 10 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Ala Phe Glu Asp Leu Arg Val Arg Ser  
1 5 10

## ( 2 ) INFORMATION FOR SEQ ID NO:5:

### ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 4 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Pro Arg Lys Arg  
1

( 2 ) INFORMATION FOR SEO ID NO.6:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 9 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( X 1 ) SEQUENCE DESCRIPTION: SBQ ID NO.6:

Val Ser Arg Lys Arg Pro Arg Pro Ala  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:7:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 6 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ala Pro Thr Lys Arg Lys  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:8:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 5 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( X 1 ) SEQUENCE DESCRIPTION. SEQ ID NO 8.

Lys Arg Pro Arg Pro  
1 5

( 2 ) INFORMATION FOR SBQ ID NO-9

( 1 ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 7 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Pro Met Lys Lys Lys Arg Lys  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:10:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 17 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( 2 ) SEQUENCE DESCRIPTION: SEQ ID NO-10:

Arg Pro Ala Ala Thr Lys Lys Ala Gly Glu Ala Lys Lys Lys Lys Leu  
1 5 10 15

**A s p**

( 2 ) INFORMATION FOR SEQ ID NO:11:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 5 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x ) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Lys Lys Lys Ile Lys  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:12:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 19 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Arg Val Thr Ile Arg Thr Val Arg Val Arg Arg Pro Pro Lys Gly Lys  
1 5 10 15  
His Arg Lys

( 2 ) INFORMATION FOR SEQ ID NO:13:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 7 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Lys Lys Arg Ser Lys Ala  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:14:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 7 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Lys Ala Lys Arg Ser Lys Ala  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:15:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 5 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Asp Arg Leu Arg Arg  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:16:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 6 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Pro Lys Glu Lys Arg Lys  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:17:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 7 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Val Arg Lys Lys Arg Lys Thr  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:18:

( i ) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Lys Lys Ser Lys Glu Glu  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:19:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 9 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO 19:

Pro Ala Ala Lys Arg Val Lys Leu Asp  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:20:

## ( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 10 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Arg Glu Arg Arg Asn Glu Leu Lys Ser Phe  
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:21.

## ( ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 7 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( i i ) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Thr Lys Lys Arg Lys Leu Glu  
1 5

( 2 ) INFORMATION FOR SBQ ID NO-2:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 7 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x 1 ) SEQUENCE DESCRIPTION: SBO ID NO-22:

Pro Lys Thr Arg Arg Arg Pro  
1 5

## ( 2 ) INFORMATION FOR SBO ID NO-23:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 7 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SBQ ID NO-23:

Ser   Ola   Arg   Lys   Arg   Pro   Pro  
               5

( 2 ) INFORMATION FOR SEQ ID NO:24:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 12 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:24:

Arg Leu Pro Val Arg Arg Arg Arg Arg Val Pro  
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:25:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 5 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gly Arg Lys Lys Arg  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:26:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 13 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Val Arg Thr Thr Lys Gly Lys Arg Lys Arg Ile Asp Val  
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:27:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 5 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Arg Lys Phe Lys Lys  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:28:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 8 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Arg Arg Asn Arg Arg Arg Arg Trp  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:29:

- ( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 15 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:29:

Pro Arg Ser Gly Lys Lys Arg Lys Arg Lys Arg Leu Lys Pro Thr  
1 5 10 15



( 2 ) INFORMATION FOR SEQ ID NO:30:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 5 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:30:

Lys Lys Lys Lys Lys  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:31:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 5 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Pro Pro Lys Lys Arg  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:32:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 6 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:32:

Pro Lys Lys Lys Lys Lys  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:33:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 9 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Ser Lys Arg Val Ala Lys Arg Lys Leu  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:34:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 8 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:34:

Pro Leu Leu Lys Lys Ile Ile Glu  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:35:

( i ) SEQUENCE CHARACTERISTICS:  
( A ) LENGTH: 8 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Pro Pro Glu Lys Lys Ile Lys Ser  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:36:

- ( i ) SEQUENCE CHARACTERISTICS:  
    ( A ) LENGTH: 7 amino acids  
    ( B ) TYPE: amino acid  
    ( C ) STRANDEDNESS: single  
    ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:36:

Pro Gln Pro Lys Lys Lys Pro  
1                    5

( 2 ) INFORMATION FOR SEQ ID NO:37:

- ( i ) SEQUENCE CHARACTERISTICS:  
    ( A ) LENGTH: 18 amino acids  
    ( B ) TYPE: amino acid  
    ( C ) STRANDEDNESS: single  
    ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Phe Lys Arg Lys His Lys Lys Asp Ile Ser Gln Asn Lys Arg Ala Val  
1                    5                    10                    15  
  
Arg Arg

( 2 ) INFORMATION FOR SEQ ID NO:38:

- ( i ) SEQUENCE CHARACTERISTICS:  
    ( A ) LENGTH: 9 amino acids  
    ( B ) TYPE: amino acid  
    ( C ) STRANDEDNESS: single  
    ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:38:

Ser Lys Cys Leu Gly Trp Leu Trp Gly  
1                    5

( 2 ) INFORMATION FOR SEQ ID NO:39:

- ( i ) SEQUENCE CHARACTERISTICS:  
    ( A ) LENGTH: 8 amino acids  
    ( B ) TYPE: amino acid  
    ( C ) STRANDEDNESS: single  
    ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Gly Lys Arg Lys Asn Lys Pro Lys  
1                    5

( 2 ) INFORMATION FOR SEQ ID NO:40:

- ( i ) SEQUENCE CHARACTERISTICS:  
    ( A ) LENGTH: 7 amino acids  
    ( B ) TYPE: amino acid  
    ( C ) STRANDEDNESS: single  
    ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:40:

Lys Thr Arg Lys His Arg Gly  
1                    5

( 2 ) INFORMATION FOR SEQ ID NO:41:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 7 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Lys His Arg Lys His Pro Gly  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:42:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 18 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( 1 ) SEQUENCE DESCRIPTION: SEQ ID NO 42.

Arg Arg Leu Ala Gln Asa Arg Glu Ala Ala Arg Lys Ser Arg Leu Arg  
1 5 10 15  
Lys Lys

( 2 ) INFORMATION FOR SEQ ID NO-4:

## ( ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH 16 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Lys Lys Arg Ala Arg Leu Val Arg Asa Arg Glu Ser Ala Gln Leu Ser  
1 5 10 15

( 2 ) INFORMATION FOR SEQ ID NO.44:

## ( 1 ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 5 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( 1 ) SEQUENCE DESCRIPTION: SEQ ID NO-44:

Arg Glu Arg Lys Lys  
1 5

( 2 ) INFORMATION FOR SEQ ID NO:45:

( i ) SEQUENCE CHARACTERISTICS:

( A ) LENGTH: 18 amino acids  
( B ) TYPE: amino acid  
( C ) STRANDEDNESS: single  
( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:45:

Arg Lys Arg Lys Glu Ser Asn Arg Glu Ser Ala Arg Arg Ser Arg Tyr  
1 5 10 15  
Arg Lys

( 2 ) INFORMATION FOR SEQ ID NO:46:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 13 amino acids  
 ( B ) TYPE: amino acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:46:

Lys Lys Asn Glu Lys His Lys Leu Lys Met Lys Arg Lys  
 1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:47:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 18 amino acids  
 ( B ) TYPE: amino acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Lys Arg Pro Arg Glu Asp Asp Asp Gly Glu Pro Ser Glu Arg Lys Arg  
 1 5 10 15  
 Glu Arg

( 2 ) INFORMATION FOR SEQ ID NO:48:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 17 amino acids  
 ( B ) TYPE: amino acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Lys Leu Arg Pro Glu Asp Arg Tyr Ile Glu Thr Glu Lys Tyr Gly Arg  
 1 5 10 15  
 Arg

( 2 ) INFORMATION FOR SEQ ID NO:49:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 15 amino acids  
 ( B ) TYPE: amino acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:49:

Lys Thr Lys Tyr Gly Ser Asp Thr Glu Ile Lys Leu Leu Ser Lys  
 1 5 10 15

( 2 ) INFORMATION FOR SEQ ID NO:50:

- ( i ) SEQUENCE CHARACTERISTICS:  
 ( A ) LENGTH: 20 amino acids  
 ( B ) TYPE: amino acid  
 ( C ) STRANDEDNESS: single  
 ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:50:

Met Glu Glu Ala Val Thr Met Ala Pro Ala Ala Val Ser Ser Ala Val  
 1 5 10 15  
 Val Gly Asp Pro  
 20

( 2 ) INFORMATION FOR SEQ ID NO:51:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 15 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Met Tyr Asn Ala Ile Leu Arg Arg Lys Leu Glu Glu Asp Leu Glu  
1 5 10 15

( 2 ) INFORMATION FOR SEQ ID NO:52:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 10 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:52:

Gly Asp Arg Arg Ala Ala Pro Ala Arg Pro  
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:53:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 10 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Met Ser Glu Arg Lys Arg Arg Glu Lys Leu  
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:54:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 13 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:54:

Met Ile Ser Glu Ala Leu Arg Lys Ala Ile Gly Lys Arg  
1 5 10

( 2 ) INFORMATION FOR SEQ ID NO:55:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 16 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:55:

Lys Arg Pro Ala Ala Ibr Lys Lys Ala Gly Glu Ala Lys Lys Lys Lys  
1 5 10 15

( 2 ) INFORMATION FOR SEQ ID NO:56:

( i ) SEQUENCE CHARACTERISTICS:

- ( A ) LENGTH: 25 amino acids
- ( B ) TYPE: amino acid
- ( C ) STRANDEDNESS: single
- ( D ) TOPOLOGY: linear

( x i ) SEQUENCE DESCRIPTION: SEQ ID NO:56:

Pro Lys Lys Lys Arg Lys Val Ser Gly Gly Gly Gly Gly Lys Lys Lys  
1 5 10 15  
Lys Lys Lys Lys Lys Lys Lys Lys Lys  
20 25

What is claimed is:

1. A transfection vector comprising a synthetic polypeptide linked electrostatically to a DNA structural sequence, forming a polypeptide-DNA complex, wherein said polypeptide is comprised of (A) a polymeric chain of basic amino acid residues, (B) a nuclear localization signal (NLS) peptide and (C) a hinge region of neutral amino acids that connects said polymeric chain and said NLS peptide.
2. The vector of claim 1, wherein said polymeric chain is comprised of between 10 and 50 residues.
3. The vector of claim 1, wherein said basic amino acid residues are selected from the group consisting of lysine, arginine and ornithine.
4. The vector of claim 1, wherein said NLS peptide is selected from the group consisting of a Simian Virus 40 (SV40) large T antigen nuclear localization signal sequence, a polyoma large T antigen nuclear localization signal sequence, an adenovirus E1a nuclear localization signal sequence, and an adenovirus E1b nuclear localization signal sequence.
5. The vector of claim 1, wherein said hinge region is comprised of between 6 and 50 amino acid residues.
6. The vector of claim 1, wherein said neutral amino acids are selected from the group consisting of glycine, alanine, leucine and isoleucine.
7. The vector of claim 1, wherein said NLS peptide is located at the amino terminus of said polypeptide and said polymeric basic amino acid chain is located at the carboxyl terminus.
8. The transfection vector of claim 1, further comprising (D) a cell type-specific ligand molecule.
9. The transfection vector of claim 1, wherein said DNA structural sequence comprises (a) a segment coding for SV40 large T antigen or polyoma large T antigen and (b) a transcription factor gene.
10. A vector according to claim 1, wherein said DNA structural sequence comprises an oncogene.
11. A vector according to claim 10, wherein said oncogene is selected from the group consisting of SV40 large T antigen, polyoma large T antigen, adenovirus E1A, adenovirus E1B, v-fms, BC12, myc, and ras.
12. A vector according to claim 1, wherein said DNA structural sequence comprises a DNA sequence selected from the group consisting of a dihydrofolate reductase gene (DHFR), a thymidine kinase gene, a thymidylate synthetase gene, a DRTF1/E2F transcription factor-encoding DNA sequence, and an E2F transcription factor-encoding DNA sequence.
13. A process for producing a transformed mammalian cell line, comprising the step of transfecting a mammalian cell with a vector according to claim 1, wherein said DNA structural sequence comprises a DNA sequence selected from the group consisting of a dihydrofolate reductase gene (DHFR), a thymidine kinase gene, a thymidylate synthetase gene, a DRTF1/E2F transcription factor-encoding DNA sequence, and an E2F transcription factor-encoding DNA sequence.
14. A process for producing a transformed mammalian cell line, comprising the step of transfecting a mammalian cell with a vector according to claim 1, wherein said DNA structural sequence comprises an oncogene.

15. A transfection vector comprising a synthetic polypeptide linked electrostatically to a DNA structural sequence, forming a polypeptide-DNA complex, wherein said polypeptide is comprised of a nuclear localization signal (NLS) peptide.

16. A transfection vector wherein said transfection vector is purified and comprises a synthetic polypeptide linked electrostatically to a DNA structural sequence, forming a polypeptide-DNA complex, wherein said polypeptide is comprised of a nuclear localization signal (NLS) peptide.

17. A transfection vector in a kit wherein said transfection vector comprises a synthetic polypeptide linked electrostatically to a DNA structural sequence, forming a polypeptide-DNA complex, wherein said polypeptide is comprised of a nuclear localization signal (NLS) peptide.

18. A transfection vector outside a cell wherein said transfection vector comprises a synthetic polypeptide linked electrostatically to a DNA structural sequence, forming a polypeptide-DNA complex, wherein said polypeptide is comprised of a nuclear localization signal (NLS) peptide.

19. A process for producing a transformed mammalian cell line, comprising the step of transfecting a mammalian cell with a vector according to any of Claims 15-18.

20. A process for producing a transfected eukaryotic cell comprising the step of transfecting a eukaryotic cell with a vector according to any of Claims 15-18.

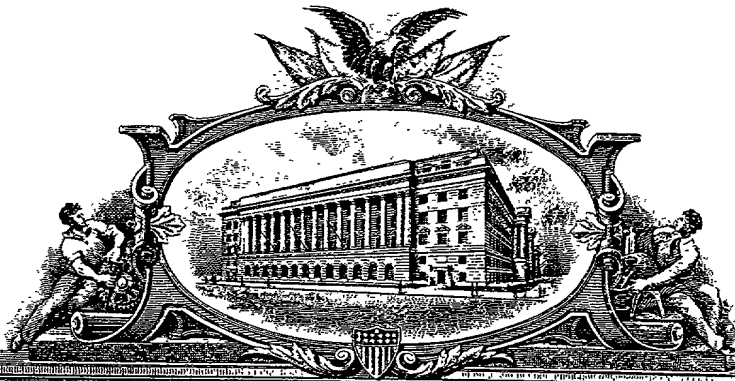
## PEPTIDE-MEDIATED GENE TRANSFER

### Abstract of Disclosure

A methodology that allows for highly efficient transfer and stable integration of DNA into both established eukaryotic cell lines and primary cells, including non-dividing cells such as human peripheral blood monocytes and macrophages, entails the use of a synthetic polypeptide comprised of a peptide domain which corresponds to a nuclear localization signal sequence and a DNA binding domain which is rich in basic amino acids, separated by a hinge region of neutral amino acid which prevents steric interference between the two domains.



PT 108533



# THE UNITED STATES OF AMERICA

**TO ALL TO WHOM THESE PRESENTS SHALL COME:  
UNITED STATES DEPARTMENT OF COMMERCE**

**United States Patent and Trademark Office**

**June 29, 1999**

**THIS IS TO CERTIFY THAT ANNEXED IS A TRUE COPY FROM THE  
ASSIGNMENT RECORDS OF ALL DOCUMENTS FOUND OF RECORD AS  
OF THE DATE OF THIS CERTIFICATION FOR:**

**APPLICATION NUMBER: 08/240,514**

**FILING DATE: May 11, 1994**

**PATENT NUMBER: 5,670,347**

**ISSUE DATE: September 23, 1997**



**By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS**

**P. SWAIN**

**Certifying Officer**

PATENT NUMBER: 5670347  
SERIAL NUMBER: 08/240514  
TITLE: PEPTIDE-MEDIATED GENE TRANSFER  
APPLICANT: GOPAL, T. VENKAT

ISSUE DATE: 09/23/97  
FILING DATE: 05/11/94

REEL: 7076 FRAME: 0100 MAILROOM DT: 06/30/94 NEW APP FIL RCPT DT: 08/08/94  
CONTROL NO. DT: 08/11/94 REC KEY DATE: 08/24/94 DT MAILED: 09/08/94  
DATE RECORDED: 06/30/94 NUMBER OF PAGES: 002

ASSIGNOR: GOPAL, T. VENKAT

EXC DATE: 06/06/94

ASSIGNEE: CLONEXPRESS, INC.  
EAST DIAMOND AVENUE, SUITE G  
GAITHERSBURG, MD 20877

50

BRIEF:

ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

06/29/99 10:04

RETURN ADDRESS: STEPHEN A. BENT  
FOLEY & LARDNER  
3000 K. STREET, N.W., SUITE 500  
WASHINGTON, D.C. 20007-5109

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DATE IN ASSIGNMENTS: 06/26/97 DT MAILED: 07/23/97  
DATE RECORDED: 06/13/97 NUMBER OF PAGES: 007

ASSIGNOR: GOPAL, T. VENKAT

EXC DATE: 05/31/96

ASSIGNEE: AMBA BIOSCIENCES  
707 STATE ROAD SUITE 201  
PRINCETON, NEW JERSEY 08540

BRIEF:

ASSIGNMENT OF ASSIGNOR'S INTEREST (SEE DOCUMENT FOR DETAILS).

06/29/99 10:04

662260-62640460

RETURN ADDRESS: ALLAN J. JACOBSON  
13310 SUMMIT SQUARE CENTER  
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LANGHORNE, PENNSYLVANIA 19047

NO MORE INFORMATION FOR THIS PATENT NUMBER 06/29/99 10:04

062260" 62540480

# RECORDATION FORM COVER SHEET PATENTS ONLY

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TO THE ASSISTANT COMMISSIONER FOR PATENTS: Please record the attached original documents or copy thereof.

1. Name of conveying party(ies): (If multiple assignors, list numerically)

AMBA Biosciences, LLC

Additional name(s) of conveying party(ies) attached?

( ) Yes (X) No

2. Name and address of receiving party(ies):

Name: Genetic Applications, LLC

Internal Address:

Street Address: 11009 Alonda Court

City: San Diego State: California ZIP: 92126

Additional name(s) of receiving party(ies) attached?

( ) Yes (X) No

3. Nature of conveyance:

- (X) Assignment  
( ) Merger  
( ) Security Agreement  
( ) Change of Name  
( ) Other:

Execution Date: (If multiple assignors, list execution dates in numerical order corresponding to numbers indicated in 1 above) July 6, 1999

4. Application number(s) or Patent number(s):

( ) Application(s) filed herewith Execution Date(s):

( ) Patent Application No.:  
Filing Date:

(X) Patent No.: 5,670,347  
Issue Date: 09/23/97

Additional numbers attached? ( ) Yes (X) No

5. Name and address of party to whom correspondence concerning document should be mailed:

Name: Nancy W. Vensko  
KNOBBE, MARTENS, OLSON & BEAR, LLP  
Customer No. 20,995

Internal Address: Sixteenth Floor  
Street Address: 620 Newport Center Drive  
City: Newport Beach State: CA ZIP: 92660  
Attorney's Docket No.: GENAPP.000GEN

7. Total fee (37 CFR 3.41): \$40

(X) Enclosed  
(X) Authorized to be charged to deposit account if any additional fees are required, or to credit any overpayment

8. Deposit account number: 11-1410

Please charge this account for any additional fees which may be required, or credit any overpayment to this account.

6. Total number of applications and patents involved: One (1)

9. Statement and signature.

To the best of my knowledge and belief, the foregoing information is true and correct, and any attached copy is a true copy of the original document.

Nancy W. Vensko  
Name of Person Signing

Signature

Date

36,298  
Registration No.

Total number of pages including cover sheet, attachments and document: 7

Mail documents to be recorded with required cover sheet information to:

Assistant Commissioner for Patents  
Box Assignments  
Washington, D.C. 20231

## Assignment of Patent Rights

THIS AGREEMENT ("Agreement"), effective as of July 6, 1999, is made by and between Genetic Applications LLC, a limited liability company ("GA") having its principal place of business at 11009 Alonda CT. San Diego, CA. 92126, and AMBA Biosciences LLC, a limited liability company ("AMBA"), having its principal place of business at 504 East Diamond Avenue, Suite G, Gaithersburg, Maryland 20877.

WHEREAS, GA is a seller, manufacturer and distributor of reagents useful in the field of molecular biology and is interested in acquiring all rights in and to United States Patent number 5670347, entitled "Peptide-Mediated Gene Transfer" ("Technology").

WHEREAS, AMBA is the sole owner of all patent rights under United States Patent number 5670347 and desires to permanently transfer all its equitable and legal rights and title in and to said patent to GA, including, but not limited to, the exclusive right to make, use, sell and license the Technology throughout the United States, and worldwide ("Territory").

NOW, THEREFORE, in consideration of the promises and the mutual covenants hereinafter set forth, GA and AMBA hereby agree as follows:

### 1. COVENANTS AND PROVISIONS

(a) AMBA does hereby sell, assign, transfer and convey all its equitable and legal rights and title in United States Patent number 5670347 to GA, and its successors or assigns.

(b) AMBA hereby represents and warrants that requests made to AMBA for use of the Technology shall be communicated to GA

immediately and that GA shall have the sole and exclusive right to license the Technology.

(c) AMBA further represents and warrants that it has investigated with due diligence its right to grant GA the rights set forth in this Agreement and believes that the use of the Technology does not infringe any patents or proprietary rights of any third party.

## 2. CONSIDERATION

(a) GA agrees to pay AMBA the amount of seven thousand dollars (\$7,000), the receipt of which is hereby acknowledged, which shall be creditable against future royalties arising from this Agreement.

(b) GA shall pay AMBA 10% of all gross income earned by GA arising from the use of Technology assigned in this Agreement. Payment shall be made to AMBA within thirty (30) days of the end of each quarter in which such income is received.

(c) GA shall pay AMBA 25% of any payment made to GA in satisfaction of money owed for use of the Technology prior to licensing. This shall include any legal or contractual settlement identified as a payment for prior infringement. Money paid under section 2(c) will not be included in calculating gross income used in 2(b). Payment shall be made to AMBA within thirty (30) days of the end of each quarter in which such income is received.

## 3. RESPECTIVE RIGHTS

(a) AMBA expressly acknowledges that all right, title and interest is to be held and enjoyed by GA and GA's successors and assigns as fully and exclusively as it would have been held and enjoyed by AMBA had this assignment not been made.

(b) AMBA expressly acknowledges that GA shall have the right to sue any party infringing upon the rights granted under United States Patent number 5670347. Further, AMBA also expressly assigns to GA the right of action for past infringements.

(c) The parties expressly agree that AMBA shall cooperate in all respects and make all relevant records, papers and information available to GA in the event of any suit GA may institute to enforce the patent rights pursuant to this agreement.

#### 4. INDEMNIFICATION

(a) GA shall indemnify, defend and hold AMBA harmless from and against any claim, liability, loss, damage, cost or expense which GA may hereinafter incur or be required to pay to a third party resulting from or arising out of any material breach of any obligations, representations or warranties hereunder.

#### 5. BOOKS AND RECORDS

(a) GA will provide AMBA with quarterly reports documenting all income from GA's license of the Technology and/or GA's sales of any Products incorporating the Technology via surface or electronic mail. Each such quarterly report shall be delivered by GA to AMBA not later than forty five (45) days from the end of the quarter to which it is attributable.

#### 6. RESOLUTION OF DISPUTES

(a) All claims or controversies between AMBA and GA under this Agreement, or the interpretation hereof, shall be resolved by final and binding arbitration in San Diego, California in accordance with the then-existing commercial arbitration rules (the "Rules") of the American Arbitration Association ("AAA"), and judgment upon the

award rendered by the arbitrators may be entered in any court having competent jurisdiction thereof; provided, however, that the law applicable to any controversy shall be the law of the State of California, regardless of its or any other jurisdiction's choice of law principles. In any such arbitration, the award or decision shall be rendered by a majority of the members of a Board of Arbitration consisting of three (3) members, one of whom shall be appointed by the AAA in accordance with the Rules. The arbitration shall take place within forty-five (45) days of the demand for arbitration. The arbitrators shall render their decision in writing to the parties hereto and their respective counsel within twenty (20) days of the completion of the arbitration.

(b) The parties consent to the exclusive jurisdiction of the courts located in San Diego CA for the resolution of all other legal controversies not expressly covered in section 6(a). The law applicable to any controversy in this section shall be the law of the State of California.

## 7. GENERAL

(a) This Agreement sets forth the entire understanding between the parties with respect to the subject matter hereof, supersedes all prior agreements, arrangements and understandings, whether written or oral. This Agreement shall be construed and enforced in accordance with the laws of the State of California applicable to contracts executed and wholly to be performed therein, without giving effect to the conflict of law principles thereof. The invalidity of any section, or provision of this Agreement shall not affect the validity of any other section, or provision of this Agreement, and each such section, or provision shall be enforced to the fullest extent permitted by law.



(b) The section headings contained herein are for reference purposes only and shall not in any way affect the meaning or interpretation of this Agreement.

(c) Nothing herein contained shall be construed to place the parties hereto in the relationship of partners, joint venturers or fiduciaries.

(d) In all aspects surrounding this assignment, both parties expressly agree to act in good faith in fulfilling their duties to the other party, as described herein.

IN WITNESS WHEREOF, the parties have caused this Agreement to be executed as of the date first printed above.

Genetic Applications, LLC.

AMBA Biosciences, LLC.

By: David R Steggs

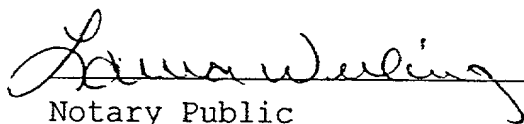
By: T. Vincent Sygal

Title: CEO

Title: President

6622630" 62640460

On this 6 day of July, 1999, before me,  
Laura Werling, the undersigned Notary  
Public, personally appeared Thiru Venkat Gopal,  
ASSIGNOR, personally known to me (or proved to me on the basis of  
satisfactory evidence) to be the person whose name is subscribed  
to the within instrument, and acknowledged to me that he executed  
the same.

  
Notary Public

LAURA WERLING  
NOTARY PUBLIC STATE OF MARYLAND  
My Commission Expires October 1, 2002

Thiru Gopal (only)

**REISSUE APPLICATION BY THE INVENTOR, OFFER TO SURRENDER  
(37 CFR 1.178)**

**TO THE COMMISSIONER OF PATENTS AND TRADEMARKS:**

The undersigned applicant of the accompanying application for the reissue of letters patent for the improvement in PEPTIDE-MEDIATED GENE TRANSFER, U.S. Patent No. 5,670,347, issued on Sep 23, 1997, of which Genetic Applications, LLC is the sole owner by assignment, and on whose behalf and with whose assent this accompanying application is made, hereby offers to surrender said letter patent.

Filed herewith is an abstract of title, duly certified, in accordance with the requirements of 37 CFR 1.171.

APPLICANT

Dated: 9/21/99

T. Venkat Gopal  
T. Venkat Gopal

**ASSENT OF ASSIGNEE TO REISSUE,  
ESTABLISHMENT OF RIGHT OF ASSIGNEE TO TAKE ACTION AND  
REVOCATION AND POWER OF ATTORNEY**

The undersigned is empowered to act on behalf of the assignee, Genetic Applications, LLC ("Assignee").

In accordance with 37 CFR 3.73(b), Assignee represents that it is the assignee of the entire right, title and interest in the above-identified letters patent. Ownership by the assignee is established as follows. A chain of title from the inventor to the current assignee is shown below:

1. From: T. Venkat Gopal  
To: CLONEXPRESS, INC.  
An assignment which was recorded in the PTO at Reel 7076, Frame 0100.
2. From: T. Venkat Gopal  
To: AMBA BIOSCIENCES  
An assignment which was recorded in the PTO at Reel 8558, Frame 0721.
3. From: AMBA BIOSCIENCES, LLC  
To: GENETIC APPLICATIONS, LLC  
An assignment which was separately submitted for recordal, a true copy of which is attached.

The undersigned has reviewed all the documents in the chain of title of the letters patent identified above, and to the best of the undersigned's knowledge and belief, title is in the Assignee.